MULTIPLE SCLEROSIS:
An Analysis of T-Cell Subsets in CNS Plaques With Attention to Plaque Borders

Kimberli Etta McCallum

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ABSTRACT

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Using monoclonal antibodies and an immunoperoxidase technique, this laboratory previously demonstrated a predominance of the cytotoxic-suppressor T-cell subset in a sampling study of plaque and non-plaque areas in C.N.S. sections (4). The margin itself was not traversed. Pan T-cells (OKT3) and both the cytotoxic-suppressor (C-S) and helper-inducer (H-I) subsets were counted in representative plaque and non-plaque areas to compare active and inactive plaques. Non-plaque areas associated with active plaques had fewer C-S cells than areas associated with inactive plaques ($p < .05$). The sum of H-I and C-S cell subsets was compared to the number of pan T-cells in both active and inactive plaques and in non-plaque areas. C-S cells were more numerous than H-I cells or pan-T-cells.

The present investigation was designed to test the hypothesis that T-cells have a special relationship to the

borders of MS plaques. This report offers a quantitative analysis of the distribution of helper-inducer cells (H-I, LEU3A +) and cytotoxic-suppressor cells (C-S, OKT8 +) in a standardized region surrounding the plaque edge (15 sections, 9 cases). In addition, every field in three complete sections from three separate cases was counted (2 active, 1 inactive by histopathologic criteria). C-S, H-I, and pan T-cells were enumerated in each of the border, plaque, and non-plaque regions for comparison.

Graphs generated from the study of the border showed that both the cytotoxic-suppressor and the helper-inducer subset peaked directly on the edge ($p < 0.01$). Pan T-cells and both subsets were found to cluster at the border region in the three whole plaques. Regional variation in the relative numbers of T cells was observed. The sum of the two T-cell subsets was significantly greater than the number of cells bearing the pan T-cell marker in two plaques and in two border regions. The C-S:H-I was greater in the inactive plaque than in two active plaques. Both subsets were concentrated in border regions and in the interiors of active plaques with fewer C-S cells in the non-plaque regions.

The present results support a crucial role for T-cell subsets in the dynamics of plaque margins. When taken together with similar findings in experimental allergic encephalomyelitis (EAE), they lead to the tempting thesis that helper-inducer cell dominance at the border is

necessary for plaque expansion while cytotoxic-suppressor dominance in non-plaque areas is associated with controlled disease. In addition, they suggest either modulation of the OKT3 surface antigen or the presence of non T-cell/OKT8+ (natural killer cells?) in association with the site of histopathologic change.

Acknowledgements

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Introduction

Multiple sclerosis is a demyelinating disease characterized by discrete plaques scattered throughout the C.N.S. Over the last 5-10 years there has been increasing evidence that host immune mechanisms play a role in the expression and progression of MS. The nature of this involvement however has remained elusive.

Strong evidence for changes in immune function associated with MS progression was first presented by Antel et. al. in 1978 (2). Con-A activated suppressor cell activity was examined in peripheral blood lymphocytes in patients with active disease, after flare up, and in those with stable disease. The level of supression induced by Con-A activated C-S cells on mitogenic responses of peripheral blood lymphocytes was reduced in active disease (3% +/- 8%) when compared to patients with stable MS (30% +/- 8%), patients after flare up (62% +/-5%), and controls (40 +/- 5%). In addition, the inhibitory effect of the supernatants from these activated C-S cells was reduced both in active and stable MS patients when compared to controls.

Subsequent studies showed an association between the expression of clinical MS and changes in the ratio of peripheral cytotoxic-suppressor (C-S) cells to helper-inducer (H-I) cells . Reinherz et. al. (21) used T3 (Pan T), T4 (H-I), and T5 (C-S) antibodies to evaluate these

subsets in the peripheral blood of 33 patients with untreated MS. Their data revealed a reduced percentage of T3+ and T5+ cells in active MS. The T4/T5 ratio increased from 2-3/1 in normals to 4/1. In a study by Bach et. al. (3), T-cell subsets were evaluated in the peripheral blood of 47 patients (25 acute phase, 14 remission, 12 progressive MS). The number of OKT3+ cells was not significantly different in MS patients when compared to controls. The percentage of OKT4+ T-cells was higher in patients with acute and progressive MS than in normal subjects or those in remission. Other investigators (31) showed an increase in the T4:T8 ratio in the majority of acute patients tested early in the course of an attack and in 25-40% of chronic patients. Compston (7, 8) evaluated T cell subsets in the peripheral blood of 36 patients in different stages of clinical MS. Patients in relapse had lower levels of OKT8 cells when compared to normals (14.07% +/- 3.71% vs 29.42 +/-4.69%). No significant differences in OKT4 cells are found between the two groups. Longitudinal studies of lymphocyte populations in normal controls revealed very little variation in the number of OKT8 cells within individuals. In contrast, most patients with relapsing remitting MS had a reduced number of OKT8 cells at presentation which returned to normal range within one month from the onset of symptoms. In 25 % of the patients, abnormalities of lymphocyte populations occurred unaccompanied by new symptoms or signs. Studies of spouses

and siblings of affected patients revealed concomitant abnormalities in peripheral subsets unassociated with disease suggesting that environmental factors contributed to the susceptibility of these patients.

Newer techniques for T-cell subset identification involving combinations of subset markers may illuminate more subtle changes in peripheral T-cell populations associated with stages of clinical MS. In a recent report (19), patients studied in both stable phase and in acute relapse showed no consistent changes in peripheral T-cell subsets. Chronic progressive MS was associated with fewer LEU2A (C-S) positive cells when compared to stable MS. Fluctuations in the number of H-I cells were seen but there was no association with clinical activity. OKT5 cells were shown to be a subpopulation of OKT8 or LEU2A cells. OKT5 labelling fluctuated much more than that of the subsuming population. Apparent descrepancies in the various studies reported may be accounted for by these findings. Other investigators (23) have used monoclonal antibodies to the T-cell subset markers CD4 (H-I) and CD8 (C-S) in addition to the common leukocyte markers Lp220 and Lp95-150 to analyze peripheral blood leukocytes in active MS. A high frequency of patients with active MS (80%) had a selective reduction of the CD+Lp220+ subset compared with controls and with patients with inactive MS. The total C-S and H-I cell frequencies and the H-I/C-S ratios were not significantly different between the patients and controls.

Thus, evidence for a selective loss of a fraction of the peripheral T-helper cells in active MS has been presented. Although the relationship between a drop in peripheral C-S cells and acute MS is in doubt, substantial evidence for this associated change remains in a proportion of patients with chronic-progressive disease. The presence of asymptomatic fluctuations in the T-cell subsets in peripheral blood is well established.

Some investigators have studied the pleocytosis of the CSF sometimes associated with active MS. Weiner et. al. (29) reported that 78% of the CSF cells were T lymphocytes with a T4 to T8 ratio of 2:1. No changes in CSF phenotypes, however, were related to changes in circulating T-cells or disease activity. Hommes et. al. (16) compared T-cell subsets in the spinal fluid of untreated MS patients to subsets in the CSF of immunosuppressed (oral cyclophosphamide / prednisone) chronic-progressive MS patients. The percentage of C-S cells out of total cells was greater in the treated group than in controls or in the untreated group . In addition, the percentage of H-I cells out of total cells was lower in the peripheral blood of treated patients while the C-S percentage was unchanged. A negative correlation was found between the levels of intrathecal IgG and the percentage of C-S cells in the CSF of untreated patients that was not reflected in the peripheral blood. Thus, although T-cell abnormalities of the CSF have been shown to be associated with MS, this T-

cell population has not been shown to directly reflect changes evident in the peripheral blood.

The studies outlined above suggest that T-cells are involved in the pathogenesis of MS. They do not, however, describe the pattern of infiltration in the target organ. In an immunocytochemical study Esiri et. al. (11) reported a qualitative and quantitative analysis of Immunoglobulin (Ig) containing cells in C.N.S. sections. Ig containing cells were more numerous within plaques than in the normally myelinated parenchyma and more numerous in recent plaques than in old plaques. Further characterization of the inflammatory infiltrate associated with MS, namely the distribution of the T-cell subsets, became possible with monoclonal antibodies to T-cell subset differentiation antigens and better staining techniques for CNS sections.

In a qualitative study, Traugott et. al. reported (27) use of monoclonal antibodies combined with a peroxidase-anti-peroxidase (PAP) technique to locate total T-cells (T11), H-I cells (T4), C-S cells (T8), and Ia+ cells which fit the morphologic criteria for macrophages, in frozen sections containing MS plaques with varying disease activity. Ia+ cells were most numerous at the lesion center and decreased in number towards the edge of the lesion in active and in chronic active plaques. In active lesions, pan-T-cells, H-I cells, and C-S cells displayed a pattern similar to that of the Ia+ cells. Virtually no infiltrating cells were found in the normal

white matter. In active chronic MS, the numbers of H-I, C-S, and pan-T-cells increased from the plaque center to the plaque edge. Pan-T cells and and H-I cells penetrated into the normal white matter while C-S cells were confined to the plaque edge. Sections containing silent chronic plaques had fewer T-cells and fewer Ia+ macrophages than sections containing active and active chronic lesions.

Our laboratory reported the first quantitative study of T-cell subsets in CNS sections containing MS plaques (4). T-cells were counted in seventeen sections from ten cases using a sampling technique. Thirty high power fields were selected in the same relative white matter location in each of the sections stained with the C-S (OKT8) , H-I (LEU3A), and Pan T (OKT3) anti-subset markers. Plaque and non-plaque areas were enumerated separately. The sites chosen for plaques included the center, intermediate zone, and demyelinated portion of the margin. Non-plaque areas varied in location but avoided overlapping the plaque margin.

Differences between the number of cells bearing OKT8, LEU3A, and OKT3 markers were examined with a T test for dependent paired data. Both T-cell subsets were present in the normal appearing parenchyma and in both histopathologically active and inactive plaques. T-cells bearing the C-S differentiation antigen were found to be three times as numerous as those bearing the H-I antigen (table 1) ($p < 0.015$). Furthermore, the C-S subpopulation

	Area Sampled	
	Plaque	Nonplaque
Pan T	1.12 +/- 1.78	0.51 +/- 0.50
H-I	0.70 +/- 0.54	0.46 +/- 0.47
C-S	2.14 +/- 2.32	1.34 +/- 1.32
H-I/C-S	.33	.35

TABLE 1
T-cell subsets and ratios in plaque and non-plaque areas of 17 C.N.S. sections from 11 cases of M.S. Data is recorded as the number of antigen positive cells +/- S.D. per high power field (X400). Thirty high power fields were counted for each region.

was more numerous than the population bearing the pan-T-cell marker. The same level of significance was achieved for this comparison. Under the same conditions in other tissues (spleen, tonsil, and lymph node) appropriate equivalence between the sum of the subsets and the number of cells bearing the pan-T-marker was noted.

Active and inactive plaques were compared with respect to T-cell populations in plaque and non-plaque regions. As shown in table 2, the number of H-I cells was very similar in active, or inactive plaques, and in all nonplaque areas. The number of C-S cells in plaque areas was also not obviously related to the plaque activity. However, in non-plaque areas associated with active plaques there were considerably fewer C-S cells than in non-plaque areas associated with inactive plaques. When these data were compared with an independent random sample T test for unequal variances, the difference was found to be significant at $p < 0.05$.

Other investigators emphasize the association of S-C cell infiltration with MS. In a recent study of over 30 MS brains (30) a similar distribution of T-cells in regions of perivascular cuffing and in CNS parenchyma with a predominance of C-S cells over H-I cells and numerous Ia+ macrophages was reported.

EAE

Animal models also point to a role for T-cells in the

	ACTIVE		INACTIVE	
	6 cases, 6 sections		5 cases, 11 sections	
	Plaque	Non-plaque	Plaque	Non-plaque
Pan T	1.79 +/- 2.45	0.35 +/- 0.20	0.75 +/- 1.28	0.59 +/- 0.60
H-I	0.81 +/- 0.49	0.41 +/- 0.55	0.65 +/- 0.59	0.49 +/- 0.45
C-S	2.33 +/- 2.69	0.64 +/- 0.53	2.04 +/- 2.22	1.72 +/- 1.48
H-I/C-S	0.35	0.64	0.32	0.28

TABLE 2

T-cell subsets and ratios in C.N.S. sections: a comparison of active and inactive plaques . Data is recorded as the number of antigen positive cells +/- S.D. per high power field (X400). Thirty high power fields were counted for each region.

pathogenesis of MS. Acute experimental allergic encephalomyelitis (EAE) is an autoimmune disease that develops in susceptible experimental animals sensitized with myelin basic protein or spinal cord homogenate and complete Freund adjuvant. In the Lewis rat, symptoms are transient and self limiting, appearing 10-15 days after innoculation and lasting 3-5 days. A chronic relapsing disease can be induced in guinea pigs and, under special conditions, in rats. The extent to which EAE is a faithful model of MS is unclear. However, EAE is an invaluable tool for studies characterizing the components of the immune system necessary for plaque initiation. The ability to predict the appearance of symptoms and monitor changes prior to clinical expression of EAE may allow demonstration of mechanisms that cannot be evaluated in MS and are essential to our understanding of active disease.

Most reports support a crucial role for T-helper cells in acute EAE. In 1960, Paterson et. al. (18) demonstrated that EAE could be transferred from sensitized donors to naive recipients by cells and not by serum. More recently, Hauser et. al. 1984 (12) showed that thymectomized irradiated mice reconstituted with Lyt 1 (H-I), Lyt 2 (C-S), or Lyt 1 + Lyt 2 developed EAE after reconstitution with Lyt 1 or Lyt 1 + Lyt 2 cells and not consistently with Lyt 2 cells alone. Thus, Lyt 1 cells (H-I) are necessary and sufficient for restoring susceptibility to EAE in mice. Others showed that pre-treatment of sensitized lymphocytes

with monoclonal antibodies against the H-I subset blocked passive transfer of clinical EAE in rats (26). These data are corroborated by reports of the treatment of acute EAE. Brostoff and Mason (5) injected W3/25, a monoclonal antibody recognizing rat T-helper cells into Lewis rats 12 to 13 days after induction. The antibody was not cytotoxic but reduced the intensity of labelling on the target cells. This treatment lead to an early recovery not accomplished with anti-pan-T-cell antibody. No qualitative histologic changes were noted after this treatment and recovery. Waldor et. al. (28) prevented the development of EAE and reversed the symptoms of mild EAE with early administration of the monoclonal antibody GK1.5 directed against the murine helper-inducer marker L3T4. In these studies, a reduction of L3T4 cells was demonstatrated in the spleen and lymph nodes. In addition, fewer perivascular infiltrates in the brains of treated animals were demonstrated. No evidence for alterations in T-cell subset balance in CNS sections associated with treatment using monoclonal antibodies against the H-I subset in EAE has been reported to date.

Immunohistopathologic studies do, however, confirm the predominant role of the helper subset. Hickey et. al. (15) used monoclonal antibodies in a quantitative study of cellular infiltration in the spinal cord of Lewis rats during acute EAE. They found that the infiltrates were predominately W3/25 cell type.

The influx of cells into the CNS compartment may be reflected in the peripheral blood in EAE. Hauser et. al. (13) demonstrated that the percentage of lyt 1 cells (H-I) cells in the peripheral blood was transiently reduced during the immediate pre-symptomatic phase of EAE . Immunohistochemical analysis of CNS infiltrates revealed that the earliest lesions consisted of predominately Lyt 1+2- lymphocytes. Hickey et. al. (14) attempted to distinguish between pathologic changes in the chronic relapsing disease and the acute self-limited form. They found that the major differences between the T-lymphocyte populations in acute EAE compared to the asymptomatic condition were an increase in the OX8 (C-S) T-cells and a diffuse scattering of lymphocytes in the CNS in the asymptomatic states. Taken together, the EAE studies demonstrate a requirement for H-I T-cells for disease induction and an association of C-S T-cells with inactivity.

Hypothesis

The above studies have attempted to describe the inflammatory changes present in the peripheral blood, CSF, and CNS associated with clinical MS and EAE. Most histopathologic reports have discussed general changes in the infiltration of the CNS parenchyma or in perivascular regions. As the plaque edge may be the locus for immune interactions which control lesion progression, subset

distribution in this defined area may shed light on the mechanisms which lead to plaque progression or hold plaques in check. This thesis provides the first quantitative analysis of T-cell subsets in the region surrounding the plaque edge. If T-cells are involved in the pathogenesis of MS they must have a role at the plaque edge.

My hypothesis is that T-cells are not randomly distributed in the CNS but, instead, have a special relationship to the plaque border. I have examined the distribution of T-cell subsets about the plaque edge, differences in this distribution associated with plaque activity, and the predicted verses the observed distribution of cells bearing the pan-T-cell marker in the CNS. I hope that these findings will contribute to our understanding of the pathogensis of MS and thus provide guidance towards specific rational therapeutic intervention.

MATERIALS AND METHODS

Tissue Preparation and Staining Methods

As previously reported, material for study was obtained from the UK and from the National Neurologic Specimen Bank in the United States.(4) For the present study, CNS material from 10 cases (17 sections) were studied. Tissues from the UK were processed fresh while tissues from the National Neurological Specimen's Bank were received frozen. Sections were cut with a cryostat (-20 degrees celsius) at 10-12 um thickness, mounted on gelatin coated slides, and fixed in acetone for ten minutes. All sections were brought to room temperature and washed with TRIS buffer (pH 7.4) and were treated with monoclonal antibodies diluted 1:5 or 1:10 in TRIS buffer. Serial sections were treated with LEU3A antibodies (Becton Dickson) against helper-inducer subset antigen, OKT8 antibodies (Ortho Diagnostics) against the cytotoxic suppressor subset antigen, and OKT3 antibodies against the pan T-cell antigen (Ortho Diagnostics). After incubation for 45 minutes in a humidified atmosphere , the sections were washed and exposed to peroxidase conjugated rabbit antibody against mouse immunoglobulin (Dako) for thirty minutes and washed again before treatment with diaminobenzidine (60 mg/100 ml in TRIS buffer) with one drop of H2O2 for five minutes. Sections were then washed in water, lightly counterstained with heamatoxylin blue,

dehydrated, cleared, and mounted. (Figure 1A & B) Separate sections from each block were also stained with a modified hematoxylin stain and Oil Red O so that the activity of the plaque could be accessed. The heamatoxylin stain demonstrated the boundary and the cellularity of the lesion whereas the Oil Red O stained phagocytic cells laden with the products of myelin breakdown. Stained specimens were scanned at intermediate power so that the exact edge could be marked.

Method of Counting of the Border Region

Sections were selected for determination of the distribution of the T-cell subsets in an area surrounding the border of a typical MS plaque. Fifteen sections from nine cases were suitable for this method. The borders of plaques stained with the LEU3A and OKT8 markers were carefully defined by comparing them to the sections stained with modified heamatoxylin. A glass piece with square grids was fitted into the eyepiece of the microscope to facilitate counting. Cells positively stained with each antibody were counted in square grids that were each circumscribed by one high power field (X400). Data was recorded as the number of positively stained cells within a high power field grid. One field was centered on the border and the number of positive cells was recorded. Three fields into and three fields away from the plaque were also counted. Another field, centered adjacent to the

A

B

FIGURE 1

These color photographs were reproduced from transparencies. They demonstrate immunoperoxidase staining in spinal cord sections. Each is lightly counterstained with heamatoxylin blue.

A. LEU3A+ stained cells in the parenchyma near the plaque edge. 250X magnification.

B. OKT8+ stained cells in the parenchyma surrounding two perivascular regions. 250X magnification.

first border field, was then centered on the border and the counting process into and away from the plaque was performed. This was repeated up to six times to produce a series of seven field vectors for each plaque studied (Figure 2A and B). Equivalent areas of the border were tabulated for each of the two antibodies. To estimate the number of positive cells at each of the seven distance fields we had to first take into account large differences due to the variable number of positive cells in a section. This regression problem was soluble with Generalized Linear Integrative Modelling (GLIM). GLIM is a program designed to address non-orthogonal multiple regression problems (3.12<C> 1977 Royal Society, London). Because the average number of T-cells per field was low, the distribution was assumed to be poisson and corrections were made for deviations.

Estimation of Histopathological Activity

It seemed possible that the distribution of T cell subsets about the border would vary with the histopathologic activity. This was scored on the basis of three criteria: the presence of macrophages, degree of cellularity, and the presence of Oil Red O phagocytosed material. Eight border regions were inactive (score=1), four showed some signs of activity (score=2), and three were considered to be active (score=3) in the border study.

In the study employing sampling of plaque and non-

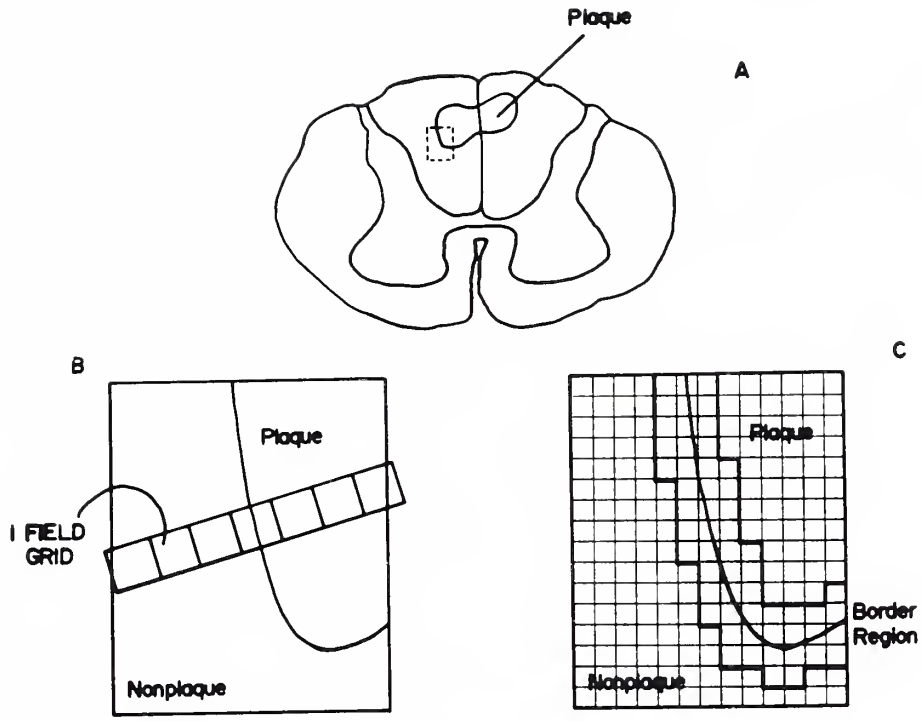

FIGURE 2

Techniques for the quantitative evaluation of plaque borders.

A. Schematic diagram of a M.S. plaque in a typical C.N.S. section (spinal cord).

B. Close up of the plaque edge: technique for the border study. One square field grid is centered on the plaque edge and 6 flanking fields were counted. This process was repeated several times along various sections of the plaque edge.

C. Close up of the plaque edge: technique for counting 3 whole plaques. Every field in the section is counted and recorded in a grid. The plaque edge is projected onto the plaque and fields falling into a predefined border region were identified.

plaque regions preceeding this work , it was not possible to score activity as precisely because the larger regions encompassed were not uniform with respect to histopathologic activity. Therefore, only the predominance of activity or inactivity was considered. Six sections were considered active and eleven inactive.

Counting of Whole Sections

Three plaques from three different cases were selected in which the staining and the tissue presentation of comparable sections stained with the OKT8, LEU3A, and OKT3 T-cell markers were uniform throughout the sections. The number of positive cells were tabulated for every field in the sections. This resulted in three data maps for each section composed of squares of equal size recording the number of positive cells found in each field. The border lines of the plaques were drawn by projection. Fields that were transected by a plaque edge as well as the adjacent whole fields were counted as "border area". The remaining fields were counted as plaque or non-plaque areas (Figure 2A and C). All fields in each area were added together and the mean numbers of positive cells per field were calculated.

RESULTS

T-cell Subsets on the Border

Scanning the sections under low power, it was noted that the number of immunoperoxidase positive cells varied greatly among the sections. While many sections had only a few positive cells, one had up to twenty-five positive cells in a high power field grid. Lymphocytes and macrophages were abundant in perivascular and vessel associated areas in many sections (Figure 1B). In some plaques there appeared to be a gradient of cells from the vessel decreasing outward and leading towards the border. Within each section, cells were clustered on the border. A summary of the tallies obtained in collecting the data is provided in appendix A. To quantify the number of cells with respect to the distance from the border it was necessary to consider the variability of the number of positive cells in each section. Computer estimates for the mean number of T-cells of both subsets in each distance field were derived using the GLIM program and are shown in Figure 3. These estimates include both active and inactive plaques. Although some plaques had many positive cells, the average was less than two cells per high power field. Both subsets peaked on the exact border and the number of cells fell off to lower values on either side. LEU3A cells immediately inside the plaque edge fell off more quickly than in the parenchyma immediately outside of the plaque

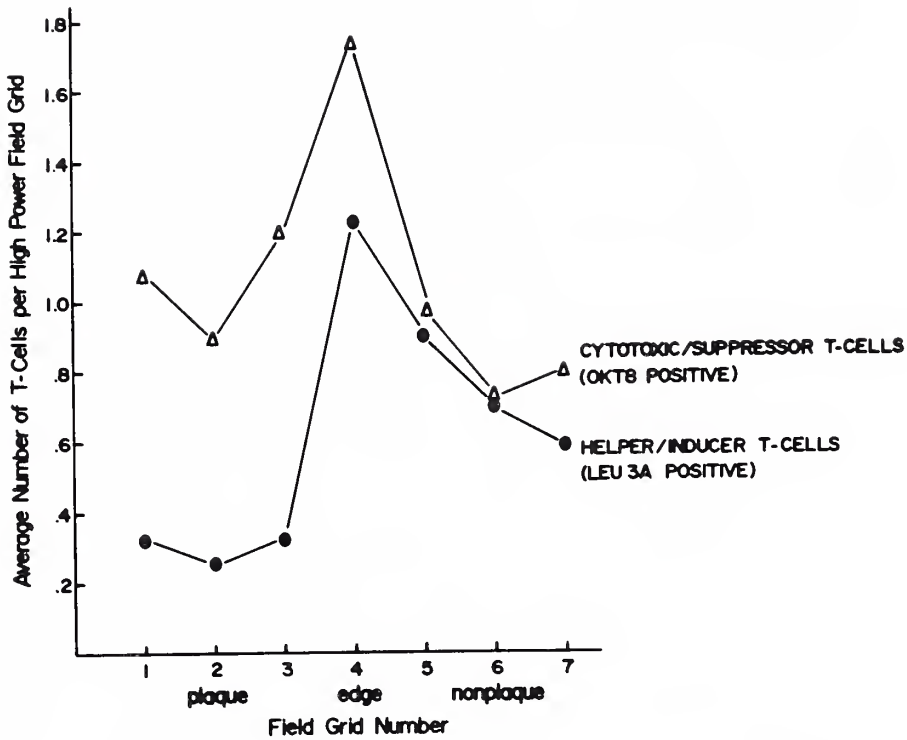

FIGURE 3

The distribution of subsets across the plaque edge. The vertical axis represents the number of cells per high power field grid estimated at each distance field using linear regression (GLIM). LEU3A cells are not distributed randomly across plaque borders ($p < 0.001$) nor are OKT8 cells distributed randomly across plaque borders ($p < .001$). OKT8 positive cells and LEU3A positive cells are more numerous in fields centered on the exact edge than in the flanking border fields. ($p < .05$ for C-S and $p < .025$ for LEU3A).

edge. There was a highly significant difference in the number of cells among distance fields across the border. ($p < .001$ for both OKT8 and LEU3A). OKT8 positive cells and LEU3A positive cells are more numerous in fields centered on the exact edge than in the flanking border fields. ($p < .05$ for C-S and $p < .025$ for LEU3A) On the whole, the cytotoxic-suppressor cells predominated. The C-S:H-I ratio was greatest in flanking fields within the plaque where it was 3:1. The raw data are included in appendix A.

Analysis by Histopathologic Activity

Since some studies have reported a reduction in the number of circulating cytotoxic-suppressor T-cells associated with MS activity (3,7,31), we were interested in any association between histopathologic activity and the distribution of T-cell subsets in the CNS.

By definition, there tended to be more cells in the border fields of active plaques. There were too few histopathologically active plaques, however, to reliably determine the effect of this factor in the border area studies. Further, activity was confounded with the variability in the number of cells in sections between individuals. One of the most active plaques had many more H-I than C-S cells as well as more positively staining cells overall. This ,however, was the only plaque in the border study in which the H-I subset predominated.

Counts of Complete Sections

All previous studies of T-cell subsets in M.S. lesions have used qualitative methods or used sampling techniques for quantitation. I have used a vector method of sampling the border region in the border study described above. In this study , by broadly defining a border area, we were able to compare the edge to fields beyond the six field grids flanking the edge. In addition, the technique of considering the border separately was applied in order to reveal the absolute distribution of T-cell subsets in three whole sections. The method of counting is shown in Figure 2C. The raw data is tabulated in appendix B.

Figure 4 compares the number of C-S cells to the number of H-I cells per high power field grid of three plaques. Sections La 34 and La 25 are active and section La 29 is inactive. The plaque edge includes the border field and two flanking fields (compare figure 2B & 2C). The plaque area showed a relative clustering of T-cell subsets. The average number of C-S cells per high power field was greater in the plaque than in the non-plaque areas in all three sections. LA 29, the inactive plaque, had the greatest number of C-S cells and the greatest ratio of C-S to H-I cells in all three regions. Furthermore, the numbers of C-S and H-I T-cells in LA 34, an active plaque, were nearly equal.

The average number of pan-T-cells has been found to be less than the number of C-S cells in samples of plaque and

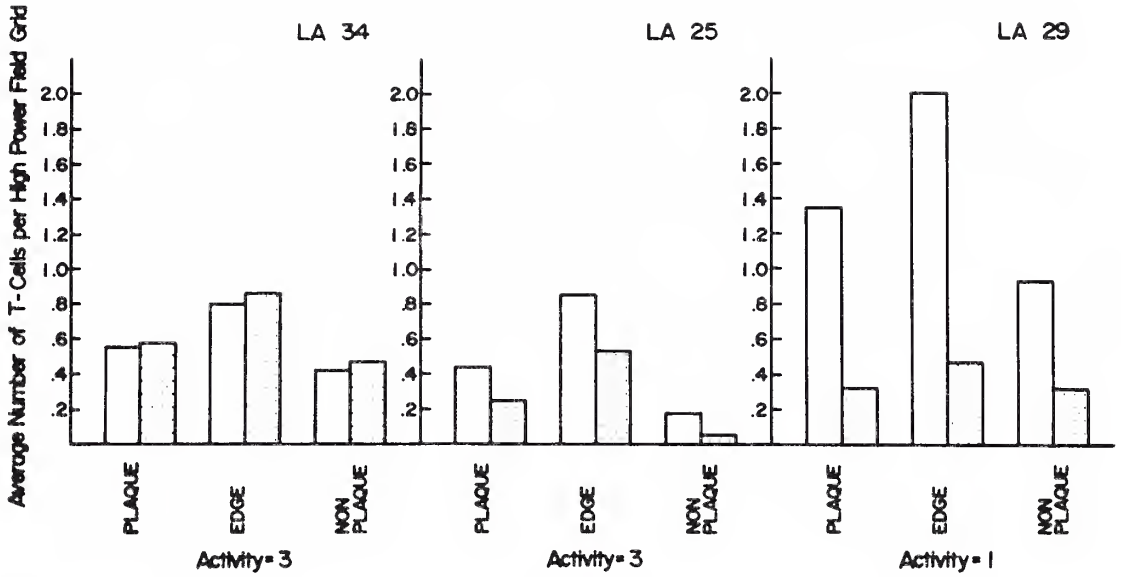

FIGURE 4
The comparison of T-cell subsets in 3 whole plaques.
KEY: /:::/ = H-I or LEU3A , / / = C-S or OKT8
Activity 3 = active; Activity 1 = inactive

non-plaque regions (table 1). The pan T-cells as well as the T-cell subsets were therefore counted in the three whole sections with particular interest in any association of this discrepency with the plaque border. Figure 4 compares the sum of the C-S and H-I T-cells to the number of cells bearing the pan T-cell marker in plaque, non-plaque, and in border areas. The sum of the subsets is greater than the number of pan T-cells in two plaque and in two border regions. The section (LA 34) with significant discrepencies in two regions is active. It has the greatest proportion of the H-I subset with a few more H-I than C-S cells in each of the three regions .

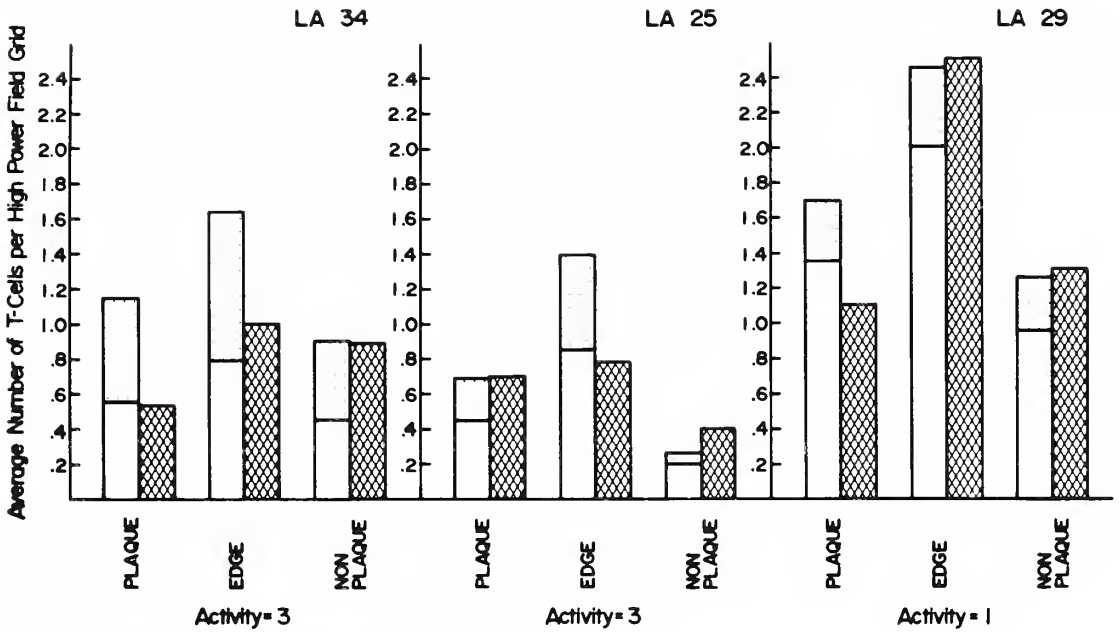

FIGURE 5

A comparison of the sum of the subsets to the number of pan T-cells in 3 whole plaques. OKT8 + LEU3A > OKT3 in 2 border regions and in two plaque regions.

Key: /:::/ = H-I, / / = C-S, /###/ = Pan T-cell (OKT3)

Activity 3 = active, activity 1 = inactive

DISCUSSION

Multiple sclerosis has a characteristic pathology marked by focal periaxial demyelination. Cases usually come to necropsy in the chronic stages of illness, death often occurring because of a secondary infection. Therefore, plaques of varying age will be observed throughout the white matter of the individual. Little is known of the initial stages of plaque expansion as we lack a sufficient amount of pathological material taken at the onset of clinical disease or relapse. Classically, lesions are thought to begin with structural changes in the myelin sheath and macrophage proliferation. Shortly thereafter, astrocytes appear to be reactive and microglia become phagocytic and may harbor products of myelin breakdown . Myelin staining reveals a clearly defined edge. Macrophages and astrocytes are more numerous throughout plaques; oligodendrocytes are less numerous. The greatest increase in cellularity has been associated with the edge of the lesion. The plaque edge, the interface between plaque and normal myelin, is the immunologic environment in which plaque expansion occurs.

Several investigators have previously studied lymphocytes associated with MS plaques. Plasma cells have been demonstrated to be associated with the edge of active plaques (11). In addition, areas of lymphocytic cuffing associated with small vessels in and around plaques have

been shown to contain many T lymphocytes (11,30). Traugott (27) found H-I and C-S cells increased in numbers from the center to the edge of active chronic lesions and H-I cells penetrated into the normal parenchyma. This laboratory has previously demonstrated a predominance of C-S cells throughout sections containing MS plaques. Weiner et. al. (29) also noted an over-representation of C-S cells compared to H-I cells in the cellular infiltration of the parenchyma as well as in regions of perivascular cuffing. The present studies were designed to evaluate the distribution of T-cell subsets about plaque borders.

I have found that both the C-S and the H-I subsets peaked on the exact border. However, the C-S and H-I distribution curves formed different patterns across the border . The slope of the H-I cell profile drops off more quickly inside the plaque than in normal parenchyma so that one field into the plaque is likely to have fewer cells than one field immediately adjacent to the edge in the myelinated parenchyma. In contrast , the number of C-S cells in fields immediately adjacent to the edge are nearly equivalent on either side of the border field. The relative balance of H-I to S-C inside the plaque edge is therefore different from the balance in fields adjacent to the plaque edge in the myelinated parenchyma . The C-S subset was more numerous than the H-I subset immediately inside the plaque but the two subsets were nearly equivalent in adjacent myelinated parenchyma. At distances

further from the edge fewer cells of either subset are present. With the exception of fields centering on vessels that exhibit lymphocytic cuffing, no fields have more cells of either subset than those associated with the exact edge of demyelination. The apparent local difference in the immune balance associated with the border of demyelination is difficult to interpret. It describes a non-uniform relationship between subsets and invokes the notion of opposing active roles for the two types of cells rather than one of a uniform effector response.

The domain of the plaque interior is immunologically different from that of the plaque exterior. In the sampling study, C-S cells were more numerous inside active plaques than in the surrounding normal parenchyma. In the study of three whole plaques including 2 active and 1 inactive plaque, C-S cells were more numerous in plaques and in the border region than in normal parenchyma. It appears that C-S cells are concentrated in border regions and in the interiors of active plaques but are more uniformly distributed in inactive plaques. Mature B cells may be distributed similarly. Esiri found that Ig containing cells were more numerous within plaques than in the surrounding parenchyma and more common in active plaques than in inactive plaques (11). On closer examination, plaque sampling reveals a non-significant predominance of H-I cells within plaques over those in the surrounding parenchyma which is again stronger in active

plaques. Taken together these findings reveal an association of plasma cells, C-S cells, and possibly H-I cells with plaque interiors and suggest that a stimulus for immune cell proliferation resides within active plaques or in the border region. As the plaque can be thought of as a sum of aging borders, the association of these cells with the borders is of particular interest.

The average number of T-cells per plaque is small and varies greatly. In the CNS of patients with no known CNS disease, the average number of LEU3A cells was .13 while the average number of OKT8 cells was .26 in each field (4). The number of C-S and H-I cells on the exact border of all MS plaques (inactive and active) that were studied was in the range of 1 to 2 cells. Many factors might effect the numbers of T cells including plaque activity, present or past therapies, and the proximity of the plaque to vessels. It is not clear whether only a few T cells need to be involved in the process or whether large numbers of T-cells are present for a brief period of time not adequately described by these data. The small absolute number of cells might reflect a regulatory rather than effector role for these lymphocytes in a scenario in which active demyelination is achieved by other cells or factors.

I suspect that present methods for identifying active plaques confound the data and obscure possible interpretations. Ideally , the criteria used to identify active plaques would divide plaques into dynamic stages of

initiation, expansion, and stabilization. Plaque oil red o positivity is a relatively late change reflecting myelin breakdown and phagocytosis. Macrophages and hypercellularity which are markers of inflammation appear early but persist. Glial scarring and hypocellularity are markers of inactivity. Unfortunately, the present criteria cannot reliably characterize a particular plaque as belonging to one of these three dynamic phases. In addition, no link between markers of activity and clinical expression of MS has been established. Therefore, comparison of T cell patterns with stages of activity defined by present histopathologic criteria is problematic and formulation of any dynamic model must be tentative.

With the above limitations in mind, our data nevertheless suggests that changes in the ratio of T cell subsets may correspond to changes in plaque activity. In our previous studies, plaque sampling revealed that the number of H-I cells was very similar in white matter surrounding both active and inactive plaques. However, in the non-plaque areas associated with active plaques there were fewer C-S cells than in non-plaque areas associated with inactive plaques. The increased number of C-S cells in non-plaque white matter associated with inactive plaques was significant at $p < 0.015$. The border study sampled the seven border fields in a set of predominantly inactive plaques and revealed a C-S dominance at the border. H-I cells outnumbered the C-S cells in one active plaque.

Several other active plaques had nearly equal numbers of the two subsets including the two active plaques in the whole plaque study . These findings, while not statistically significant, when taken together with the statistically significant sampling study, lead to the tempting thesis that lesion progression in MS is associated with H-I T-cells, while stabilization is associated with C-S T-cells.

This thesis is strengthened by similar observations in EAE. CNS infiltration by macrophages and T cells (15,25) in early lesions in mice are associated with predominately Lyt-1 (H-I) lymphocytes (13). Hauser et al. (12) showed that mice depleted of their T-cells could be immunized to regularly develop EAE only after reconstitution with cells bearing the H-I (LYT-1) marker. Brostoff and Mason (1984) demonstrated that monoclonal antibody directed against rat helper T-cells reduced the duration of symptoms in rats treated after the appearance of clinical signs (5). In contrast, Hickey et. al. showed a persistence of OX8 cells (C-S) associated with the symptom free stage in rats (14,15).

There is evidence for a correlation between T cell ratios and the expression of other immune linked disease. Typical normal tissue ratios are not well worked out for each subset. In absence of disease, few T cells are present at all. Regional differences in the predominating cell type have been documented in chronic liver disease. C-S

cells were found to be more numerous in areas of piecemeal necrosis while cells bearing the pan-T-cell marker predominate in portal areas without piecemeal necrosis (24). Colucci et. al. (6) have described a predominance of C-S cells in chronic active hepatitis and H-I cells in chronic persistent hepatitis. In graft vs host disease, HI:CS ratio is 1:10 while in rheumatoid arthritis , HI:CS tissue ratios range from 5 to 15 : 1 (17). In chronic progressive MS, we found this ratio is 1:3 (4).

The previous finding that C-S cells outnumber pan-T-cells in sampled plaque and non-plaque regions (4) was unexpected. An attempt was made to eliminate the possibility that the predominance was due to errors in technique by the use of controls. Under the same conditions in other tissues it was found that there was an approximate equivalence between the sum of the subsets and the number of cells bearing the pan-T-marker. In addition, use of the anti SRBC receptor reagent T11A did not alter the discrepancy. Oligodendrocytes did not stain with C-S antibody in control tissues to account for the apparent excess of C-S cells compared to the pan-T-cells. In the present study of three whole plaques , subsets summed to equal pan-T-cells in some areas suggesting that the discrepancy was non-uniform. Interestingly , in this small sample , only plaque and border areas had fewer than expected cells bearing the pan-T-cell marker. This suggests that the expression of the pan-T-cell marker may

be effected by the disease process. The quantitative analysis of more border regions is required before this finding can be interpreted in the context of all MS plaques.

It is tempting to attribute the observation that the C-S cells are more numerous than the cells bearing the pan-T-marker to antigenic modulation. It is reasonable to postulate a decrease in the expression of the pan-T-cell marker in certain cytotoxic-suppressor cells. If modulation is present, its role in the pathogenesis of MS is intriguing. Modulation is known to be present in some immunodeficiency states. The lymphoid cells of most patients with combined immunodeficiency lack surface expression of T3 (22) . It has been shown that T3 modulation by anti-T3 antibody has no effect on T-cell viability but decreases cytotoxic effector function of T4 and T8 clones for up to 48 hrs. In addition, the proliferative response to IL2 was greater in these cells (1). It appears that altered antigen recognition and disturbed T-cell function are associated with T3 modulation. The findings of the present study suggest that T3 modulation may be present in areas of demyelination supporting a theory of altered T-cell function in MS.

Another paradigm, the presence of a T8 bearing non T-cell population, might explain the presence of OKT8 positive, OKT3 negative cells. For example, Hepatitis B has several correlates to MS both in the active/chronic nature

of the disease and in the patterns of tissue infiltration by inflammatory cells. Colucci reported that the sum of the subsets outnumbered the pan-T-cells in 5 out of 26 cases of chronic active hepatitis (6). In acute hepatitis, Eggink et al found LEU7+ (NK cells) and OKT8+,OKT1-,OKT3- cells predominated over OKT8+,OKT1+,OKT3+ cells (9). In contrast, Chronic hepatitis B sections had a predominance of OKT8+,OKT1+, OKT3+ cells (10). Clearly, further studies are required to determine if the cells bearing OKT8 but not OKT3 antigen are natural killer (NK) cells.

In human immature "common" thymocytes, OKT4 and OKT8 antigens and not OKT3 antigens are associated with the surfaces of cells. Mature thymocytes acquire OKT3 reactivity and segregate into OKT4 or OKT8 subsets prior to exportation into the peripheral compartment (20). Most evidence supports the notion that the CNS lymphocytes associated with MS reached their destination via the blood. It is possible, however, that these OKT3- lymphocytes represent immature cells derived from stem cells native to the CNS. Interactions with soluble factors and macrophages might then provide an alternative explanation for the observation that these lymphocytes appear to cluster in perivascular regions.

A goal in the investigation of immune cell involvement in the pathogenesis of MS is to uncover patterns which would suggest a rational approach towards therapeutic intervention. Since T-cells cluster in perivascular

regions and are present within these vessels, it is generally assumed that blood borne lymphocytes have access to the CNS in MS. Peripheral forms of immunotherapy therefore seem possible. Immunotherapy might focus on the H-I/C-S balance. The function of the C-S cells remains unknown but it is critical to clarify the significance of C-S dominance. If C-S cells have a suppressor function, the theory that C-S cells hold plaque progression in check would be supported. Immunotherapy could then be tailored to increase suppression or decrease help. If, these cells were instead primarily cytotoxic, we might attempt to decrease cytotoxic activity. The histopathologic findings in MS might suggest both a time and target for specific immunotherapy. A clear dynamic paradigm based on further studies of the functional roles of immune cell subsets in the C.N.S. is needed before a safe rational approach towards immune intervention can be offered in MS.

APPENDIX

A: Border Study Data

The data obtained in the border study is provided below. It was not possible to count the same number of fields in each section because of variations in the size, shape, and staining of plaques. Analysis required the Generalized Linear Integrative Modelling program mentioned earlier in this thesis. The distribution of the number of cells per one high power field best fits the poisson distribution. Deviation from the expected distribution were noted and the appropriate transformations were performed before analysis. Estimates for each of the increasing distance regions were plotted in figure 3.

Key: Sections are named and numbered if part of a series from one case: eg. LA, UKA, UKB, UKC . Activity is ranked from least active to most active, 1 to 3. Distance fields are recorded in three fields into the plaque and three fields into the normal parenchyma with one field centered on the plaque edge as a reference point. Negative numbers represent fields inside the plaque while positive numbers represent fields in the myelinated parenchyma. This process was repeated "n" times. Totals devided by "n" would represent the average number of cells/ high power field grid at each distance parameter in the section.

1. LA #4 Activity 1

	distance	-3	-2	-1	0	1	2	3
OKT8								
	total	11	12	17	12	1	5	5
	n	5	5	5	5	5	5	4
	average # of cells on border: 2.4							
LEU3A								
	total	3	3	2	8	1	4	0
	n	2	3	4	4	4	4	1
	average # of cells on border: 2							

2. LA # 29 Activity 1

	distance	-3	-2	-1	0	1	2	3
OKT8								
	total	4	5	2	5	2	3	4
	n	6	6	6	6	6	6	6
	average # of cells on border: .83							
LEU3A								
	total	0	0	0	0	2	0	0
	n	4	4	4	4	4	4	4
	average # of cells on border: 0							

3. LA # 25 Activity 3

	distance	-3	-2	-1	0	1	2	3
OKT8								
	total	4	7	9	9	5	0	0
	n	10	10	10	10	10	10	10
	average # of cells on border: .9							
LEU3A								
	total	2	0	0	2	0	0	0
	n	4	4	4	4	4	4	4
	average # of cells on border: .5							

4. LA # 27 Activity 2

	distance	-3	-2	-1	0	1	2	3
OKT8								
	total	13	14	9	12	1	2	1
	n	4	4	4	4	4	4	4
	average # of cells on border: 3							
LEU3A								
	total	0	0	3	4	0	0	1
	n	4	4	4	4	4	4	4

average # of cells on border: 1

5. LA #35 Activity 2

distance	-3	-2	-1	0	1	2	3
OKT8							
total	5	13	16	8	4	3	
n	2	5	5	4	4	3	

average # of cells on border: 3.2

LEU3A							
total	2	6	5	4	3	6	
n	3	3	3	3	3	3	

average # of cells on border: 1.7

6. LA #36 Activity 3

distance	-3	-2	-1	0	1	2	3
OKT8							
total	3	9	4	9	3	3	2
n	5	5	5	5	5	5	5

average # of cells on border: 1.8

NO LEU3A

7. UKA Activity 1

distance	-3	-2	-1	0	1	2	3
OKT8							
total	3	2	0	1	1	0	1
n	5	5	5	5	5	5	4

average # of cells on border: .2

NO LEU3A

8. UKB #1 Activity 1

distance	-3	-2	-1	0	1	2	3
OKT8							
total	4	0	2	5	10	6	1
n	4	5	6	6	6	6	2

average # of cells on border: .83

NO LEU3A

9. UKB #2 Activity 1

distance	-3	-2	-1	0	1	2	3

OKT8

total	2	6	11	13	10	7	4
n	1	2	2	2	2	2	1

average # of cells on border: 6.5

NO LEU3A

10. UKB #3 Activity 1

distance	-3	-2	-1	0	1	2	3
OKT8							
total	0	0	1	1	0	4	4
n	4	5	5	5	5	5	4

average # of cells on border: .2

LEU3A							
total	0	0	1	0	1	1	3
n	4	4	4	4	4	3	3

average # of cells on border: 0

11. UKC #1 Activity 1

distance	-3	-2	-1	0	1	2	3
OKT8							
total	9	6	5	16	11	8	6
n	6	6	6	6	6	6	4

average # of cells on border: 2.7

LEU3A							
total	9	2	1	8	5	17	9
n	3	3	3	3	3	3	3

average # of cells on border: 2.7

12. UKC #2 Activity 2

distance	-3	-2	-1	0	1	2	3
OKT8							
total	3	1	5	6	3	8	5
n	5	5	6	5	5	5	4

average # of cells on border: 1.2

LEU3A							
total	0	2	0	0	1	3	3
n	3	3	3	3	3	3	3

average # of cells on border: 0

13. UKC #3 Activity 1

	distance	-3	-2	-1	0	1	2	3
OKT8								
	total	0	2	5	10	6	2	8
	n	3	5	5	5	5	5	5
	average # of cells on border: 2							
LEU3A								
	total	0	0	3	2	5	2	3
	n	5	5	5	5	5	5	5
	average # of cells on border: .4							

14. UKC #4 & 5 This plaque is the fusion of two smaller plaques. Both sections were sampled and included in the following talley. Activity 3

	distance	-3	-2	-1	0	1	2	3
OKT8								
	total		13	39	54	24	17	
	n		9	9	9	9	9	
	average # of cells on border: 6							
LEU3A								
	total	6	13	15	89	59	35	17
	n	4	6	7	7	7	7	5
	average # of cells on border: 12							

15. UKC #6 Activity 2

	distance	-3	-2	-1	0	1	2	3
OKT8								
	total	13	9	9	20	12	7	4
	n	6	6	6	6	6	5	3
	average # of cells on border: 3.3							
NO LEU3A								

B: Study of Three Complete sections

The following data is graphed in figures 4 and 5. Both the vessel associated and nonvessel associated numbers are tabulated below although only totals appear on the included graphs. Data is recorded as the average number of cells per field. The n values (number of counted high

power fields) are in parentheses . Key: NVA = non vessel associated fields. Total = cells in parenchyma in NVA fields in addition to those associated with vessels . Methods of determining plaque , nonplaque, and edge regions have been described in methods section.

CASE	34	25	29
PLAQUE			
NVA			
OKT8	.43(109)	.28(306)	1.25(64)
LEU3A	.33(111)	.12(202)	.25(68)
OKT3		.35(227)	.79(71)
TOTAL			
OKT8	.55(117)	.43(325)	1.36(67)
LEU3A	.41(118)	.24(231)	.32(73)
OKT3	.50(68)	.70(254)	1.1(82)
NON-PLAQUE			
NVA			
OKT8	.37(59)	.14(216)	.74(219)
LEU3A	.42(67)	.036(222)	.24(233)
OKT3	.88(42)	.31(153)	1.1(206)
TOTAL			
OKT8	.42(62)	.19(220)	.94(235)
LEU3A	.47(70)	.05(226)	.31(245)
OKT3	.88(42)	.39(156)	1.3(228)
EDGE			
NVA			
OKT8	.65(270)	.56(110)	1.8(120)
LEU3A	.56(252)	.41(104)	.39(126)
OKT3	.98(234)	.52(129)	2.2(129)
TOTAL			
OKT8	.79(283)	.85(124)	2.0(136)
LEU3A	.83(256)	.52(108)	.46(136)
OKT3	1.0(247)	.77(135)	2.5(135)

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